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ORIGINAL RESEARCH—HEAD AND NECK CANCER

The administration of IL-12/GM-CSF and Ig-4-1BB ligand markedly decreases murine floor of mouth squamous cell cancer

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OBJECTIVE: To assess immune-based gene therapy in a murine floor of mouth (FOM) squamous cell carcinoma (SCC) model.
STUDY DESIGN: In vitro and in vivo testing of immune therapy for SCC.
METHODS: Multiple SCC lines were infected by using advRSV-interleukin-12 (IL-12) and advCMV-interleukin-12/granulocyte macrophage colony-stimulating factor (IL-12/GM-CSF) and monitored for production of IL-12 and GM-CSF. Intratumoral injections of viral vectors were administered with systemic Ig-4-1BB ligand in an orthotopic murine FOM SCC model and followed for tumor size and survival.
RESULTS: In vitro, all cell lines produced substantial levels of IL-12 and GM-CSF. In vivo, tumors treated with advCMV-IL-12/GM-CSF and Ig-4-1BBL showed a striking reduction in tumor volume (vs control $P < 0.0001$) and improved median survival (38 days vs 19 days for control, $P < 0.0001$).
CONCLUSION: Combination immune-based therapies effectively improve survival in mice bearing FOM SCC over single-modality therapy.

Head and neck squamous cell carcinoma (HNSCC) represents a major cause of morbidity and mortality in this country. The American Cancer Institution estimates that there are greater than 40,000 new HNSCC cases and more than 10,000 deaths annually in the United States alone. Despite aggressive management and recent advances in surgery, radiation therapy, and chemotherapy, locoregional recurrence may occur in up to 60 percent of patients.[1] Given the current problems faced in regards to HNSCC, there exists a need for new treatment modalities.

Cancer immunotherapy is a recent advancement in modulating the innate immune system. Although the origin of tumorigenesis is clearly linked to environmental carcinogens, its progression through a series of discrete genetic changes results in emergence of the tumor that is resistant to immune effector cells.[2-4] Recent advances in immunotherapy using gene transfer have shown promising results in several forms of cancer.[5-8] The strategies in immunotherapy include the use of both viral and nonviral vectors. Past data have shown profound results with adenoviral vector constructs containing interleukin 12 (IL-12) and granulocyte-macrophage colony-stimulating factor (GM-CSF) along with systemic activation of 4-1-BB protein (a receptor involved in activation of T lymphocytes) via experimental agonistic antibodies in other forms of cancer, including skin, metastatic colon, and hepatic carcinomas.[9-11]

Recent studies have explored adenoviral gene transfer of multiple cytokines and their antitumor effects. These factors include IL-2, IL-12, GM-CSF, herpes simplex virus thymidine kinase gene, and retinoblastoma protein. Thus far, no head and neck literature have been published regarding adenoviral gene transfer with IL-12 and GM-CSF in conjunction with Ig-4-1BB ligand (L). With recent encouraging data establishing synergistic responses of these factors in other forms of cancers, we hypothesized that there may be similar synergistic effects in HNSCC.[5,7-9] Specifically, we studied gene transfer of IL-12 and GM-CSF via intratumoral injections of adenoviral vectors with and without the use of systemic Ig-4-1BBL. The specific endpoints examined were tumor size and overall survival in our orthotopic murine model. The ultimate goal was to determine the efficacy of this therapy in HNSCC, with the intent of eventually developing a clinically applicable product for human trials.

MATERIALS AND METHODS

Cell lines

Human FaDu squamous carcinoma cells were obtained from the American Type Culture Collection (Manassas,

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VA) and maintained in minimum essential medium (MediaTech, Herndon, VA) with 2 mmol/L L-glutamine and Earle's BSS (MediaTech) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate (MediaTech), and 10 percent fetal bovine serum (Atlanta Biologicals, Lawerenceville, GA).

The murine SCC cell line SCC VII was a kind gift from Drs Richard Wong and Bert W. O'Malley Jr and was maintained in Dulbecco's modified Eagle's medium containing 10 percent fetal bovine serum with 2 mmol/L L-glutamine and 0.1 mmol/L nonessential amino acids.

Human SCC 09 cells were purchased from American Type Culture Collection and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium pyruvate (MediaTech) and Ham's F12 medium pyruvate (MediaTech) containing 1.2 g/L sodium bicarbonate, 2.5 mmol/L L-glutamine, 15 mmol/L HEPES (MediaTech), and 0.5 mmol/L sodium pyruvate supplemented with 400 ng/mL hydrocortisone (BD Biosciences, San Jose, CA) and 10 percent fetal bovine serum.

Cloning strategy to generate adenovirus expressing mGM-CSF and mIL-12 (p40-linker-p35)

The construction of replication-defective adenoviral vector has been previously reported in the literature by Chen et al.[12] Briefly, the construct was confirmed by direct sequencing. After confirmation, an IL-12 fragment containing p40-linker-p35 was released from the HindIII and NotI site. The fragment was cloned into SmaI and NotI site. After that, the BglII and BSABI fragment containing a whole-expression cassette including CMV promoter, GM-CSF, IRES, p40 flexible linker, p35, and SV40polyA was released and packaged into adenovirus shuttle plasmid pΔE1sp1A (Microbix Biosystems Inc, Canada) between BamHI and EcoRV sites. The virus was rescued by the cotransfection of 293 cells with the final plasmid constructed and pJM17 (Fig 1). Viral titers were determined by standard plaque assay to determine concentration in plaque-forming units.

In vitro cytotoxicity assay

All cell lines were plated in 24-well tissue culture treated plates at a density of 2×10^5 cells/well/500 μL. The following day, cells were treated with the appropriate multiplicity of infection (MOI) of the adenoviral vector or phosphate-buffered saline (PBS) control. Cell survival was determined via the Cell Proliferation Kit-MTT assay (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. Viral cytotoxicity was determined as a percentage of the appropriate mock infected or nontreated controls at each time point. All experiments were performed in triplicate.

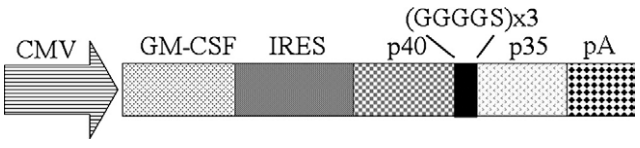

Figure 1 The expression cassette of advCMV-IL-12/GM-CSF. The internal ribosome entry site (IRES) from the encephalomyocarditis determines the translation of IL-12. Two subunits of IL-12 are linked by a flexible linker containing three repeats of GGGGS.

Protein quantification via enzyme-linked immunosorbent assay

All cell lines were plated in 48-well tissue culture plates at a density of 1×10^4 cells/well/200 μL. The following day, cells were treated with the appropriate MOI of the respective virus or PBS control. At 24 and 48 hours after infection, cell supernatants were harvested and mIL-12 and mGM-CSF levels were quantified by using the Quantikine mouse IL-12 p70 enzyme-linked immunosorbent assay (ELISA) kit and the Quantikine mouse GM-CSF kit (R&D Systems, Minneapolis, MN), respectively, according to manufacturer's instructions. Both supernatant harvests and ELISA quantifications were done in duplicate.

The assessment of in vivo intratumoral infection of murine SCC VII floor of mouth tumors

All the procedures that involved animals were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine. For the in vivo studies, we studied the FOM tumor model in immune-competent C3H/HeJ mice. This orthotopic model was chosen because it has been characterized as being aggressive and reflective of the human counterpart. On day 0, syngeneic SCC VII cells (5×10^5) were transcutaneously implanted into the FOM at the level of the mylohyoid. By day 5, tumors had grown to an average diameter of 4 to 6 mm. Daily injections were administered directly into the tumors for 5 consecutive days (days 5-9). Each tumor was injected with 2.5×10^{10} viral particles per dose of advIL-12 or advIL-12/GM-CSF. Control animals were injected with PBS; 200 μg of Ig-4-1BBL were infused intraperitoneally on days 5 and 8 in treated and control animals. The endpoints examined were the progression of tumor size and animal survival or equivalent. The Student's *t* test was used to compare virus-injected tumors with the control group as well as between groups, and the Kaplan-Meier survival curves were compared by using Graph Pad Prism 5.0 Program (GraphPad Software, San Diego, CA).

RESULTS

In vitro IL-12 expression

SCC cell lines were infected with advCMV-IL-12/GM-CSF and advRSV-IL-12 at varying MOI to quantify expression

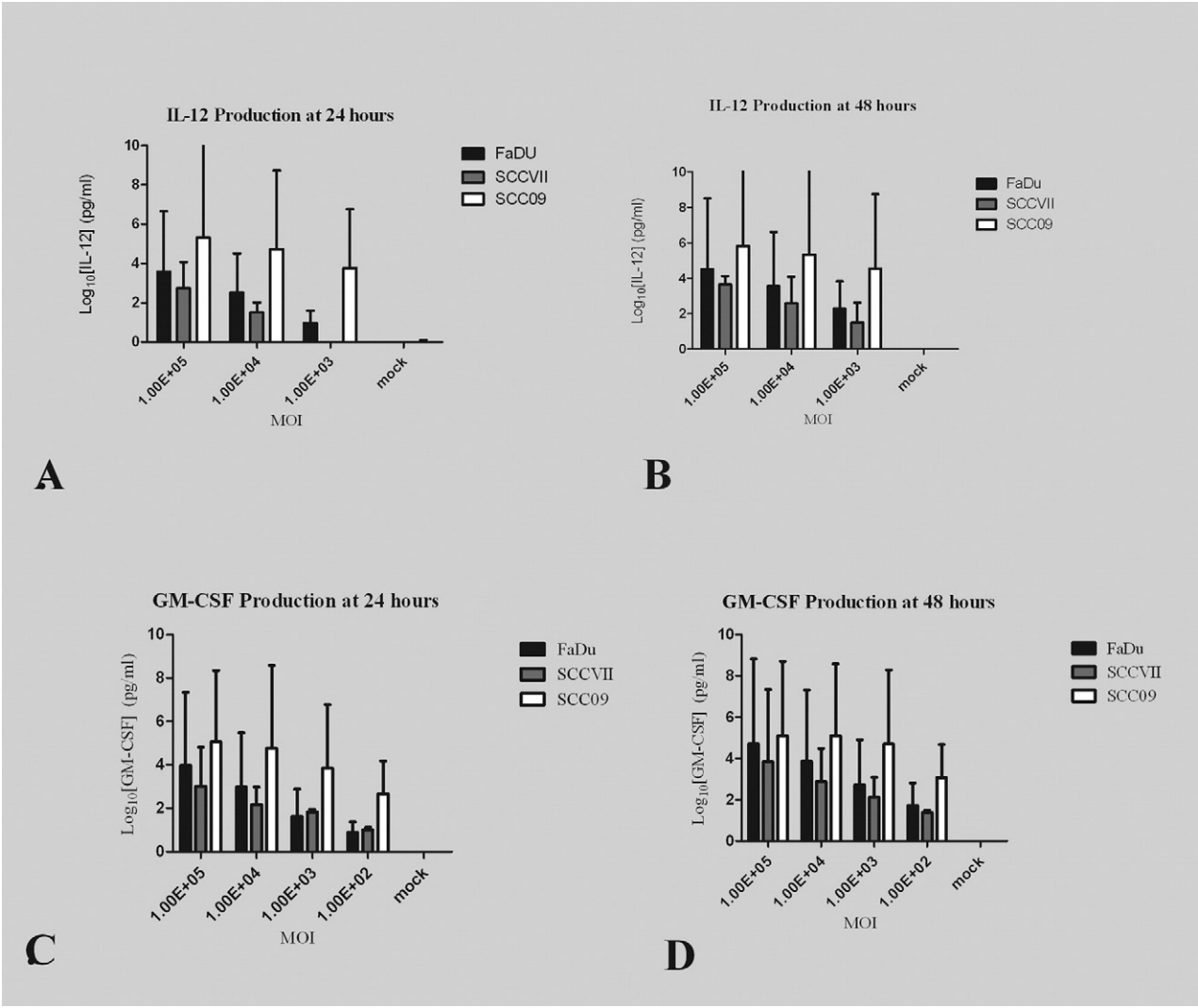

Figure 2 **(A)** In vitro IL-12 expression evaluated by a logarithmic scale at 24 hours with ELISA after infecting cell lines with advCMV-IL-12/GM-CSF. **(B)** In vitro IL-12 at 48 hours. **(C)** In vitro GM-CSF expression evaluated by a logarithmic scale at 24 hours with ELISA after infecting cell lines with advCMV-IL-12/GM-CSF. **(D)** In vitro GM-CSF expression at 48 hours.

of IL-12. After 48-hour incubation periods, murine IL-12 levels were quantified for both viral vectors. With advCMV-IL-12/GM-CSF, the three cell lines (FaDu, SCCVII, and SCC09) expressed 3.29×10^4 pg/mL, 4.40×10^3 pg/mL, and 6.66×10^5 pg/mL, respectively, at an MOI of 1.00×10^5 (Fig 2A and B). The mock-infected controls of the tumor cell lines produced no detectable level of IL-12.

Comparing the expression of IL-12 in the advRSV-IL-12 and the advCMV-IL-12/GM-CSF, there was clearly a greater expression quantified in the advCMV-12/GM-CSF group among all cell lines with an MOI of 1.00×10^3 or greater (P value <0.0001 when compared with advRSV-IL-12 as the control) (Fig 3).

In vitro GM-CSF expression

SCC cell lines were infected with advCMV-IL-12/GM-CSF at varying MOIs to quantify the expression of GM-CSF. After 24- and 48-hour incubation periods, murine GM-CSF was quantified. The human SCC cell lines showed maximal expression at 48 hours. At an MOI of 1.00×10^5, 5.46×10^2 pg/mL, and 4.4×10^3 pg/mL of GM-CSF were expressed at 24 and 48 hours, respectively (Fig 2C and D). The mock-infected control of the tumor line produced no detectable level of GM-CSF.

In vivo tumor size

The average tumor surface area (cm^2), measured by the maximal length by width, was found to be smaller in treated animals when compared at day 13. After day 13, a sizable number of animals in multiple arms died or were euthanized, altering the average tumor surface area substantially. At day 13, the control group had a mean surface area of 1.39 cm^2. In the group with adv IL-12, the average surface area was 1.03 cm^2 ($P < 0.03$ vs control) at day 13. The group treated with advIL-12 and Ig-4-1BBL had an average surface area of 0.981 cm^2 ($P < 0.013$ vs control). The group

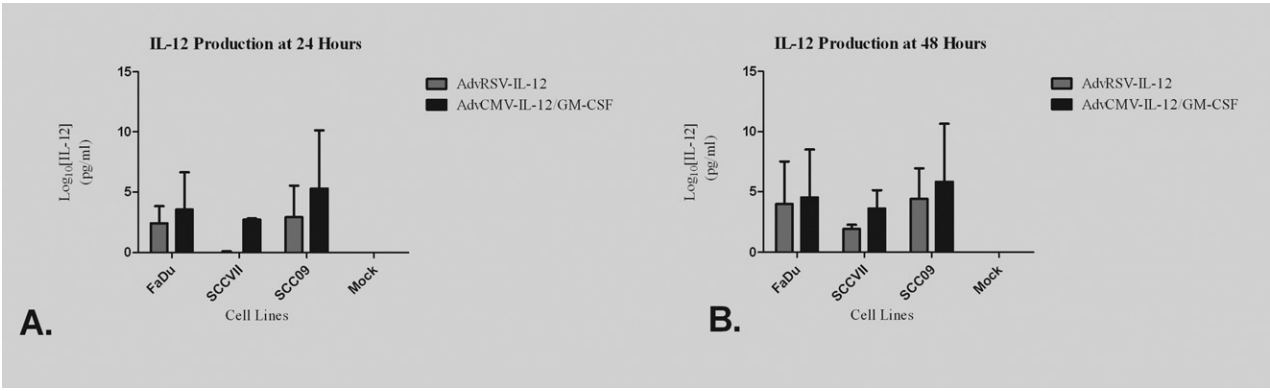

Figure 3 **(A)** In vitro IL-12 expression evaluated by ELISA at an MOI of 1.0×10^5 (maximal MOI tested) at 24 hours with both advRSV-IL-12 and advCMV-IL-12/GM-CSF on a logarithmic scale. **(B)** In vitro IL-12 expression at 48 hours. The advCMV-IL-12/GM-CSF has a greater than 10-fold increase in expression of IL-12 along all cell lines ($P < 0.001$ vs advRSV-IL-12).

with advIL-12/GM-CSF with Ig-4-1BBL had an average surface area of 0.312 cm^2 ($P < 0.0001$ vs control) (Fig 4A). When comparing the tumor size of the animals treated with advIL-12/GM-CSF and Ig-4-1BBL with the animals receiving advIL-12 and Ig-4-1BBL, the difference in average surface area was statistically significant ($P < 0.05$).

In vivo animal survival

Mice with transcutaneously implanted FOM tumors were randomly assigned to five treatment groups on postinoculation day five (n = 7-14 animals/group). The Kaplan-Meier survival curves, as shown in Figure 4B, show the death of all control group animals within 13 to 21 days after tumor implantation. The Ig-4-1BBL group had 14 percent (3/21) survive past day 21; 1 (7%) survived to day 90. The survival curve for PBS versus Ig-4-1BBL did not show a statistically significant value ($P < 0.4$ vs PBS). In the group with advIL-12, 50 percent (7/14) survived past day 21; none survived past day 39. This group also did not show a statistically significant value ($P < 0.13$ vs PBS). In the group with advIL-12 + Ig-4-1BBL, 64 percent (9/14) survived past day 21; 7 percent (1/14) survived past day 39 up to the completion of the study (day 230) and proved to be statistically significant ($P < 0.006$ vs control). In the group with advIL-12/GM-CSF with Ig-4-1BBL, 85 percent (12/14) animals survived past day 21, 50 percent (7/14) survived past day 39, and 29 percent (4/14) survived greater than 230 days after tumor inoculation ($P < 0.0001$ vs control).

When comparing the other survival curves, advIL-12 + Ig4-1BBL had a statistically significant increased survival over advIL-12 alone ($P < 0.04$ when compared with advIL-12). Similarly, advIL-12/GM-CSF + Ig-4-1BBL had a statistically significant survival advantage over advIL-12 ($P < 0.0004$). When comparing advIL-12 + Ig-4-1BBL with advIL-12/GM-CSF + Ig-4-1BBL, triple therapy improved survival over dual therapy but did not quite reach statistical significance ($P < 0.06$ vs advIL-12 +Ig-4-1BBL).

Mice in the control group had a median survival of 19 days. This was increased to 27 days in the group with advIL-12 +Ig-4-1BBL and 38 days in the group with advIL-12/GM-CSF +Ig-4-1BBL.

DISCUSSION

Recurrent disease in the field of HNSCC continues to be a significant medical problem. Despite aggressive management and recent advances in surgery, radiation therapy, and chemotherapy, locoregional recurrence may occur in up to 60 percent of patients.[1] Adenoviral-based gene immunotherapy represents a class of novel agents for cancer therapy in patients who have failed standard therapeutic regiments including surgery, chemotherapy, and radiation therapy.

Gene transfer via viral vectors had gained significant attention given its early promising results. There is an increasing body of evidence suggesting that although tumors originate secondary to environmental carcinogens, they are able to progress because they develop a resistance to the innate immune system. There are mounting data that show human tumors, in general, and head and neck cancers, in particular, have developed multiple strategies to evade and escape the host immune system. Additionally, they exert profound inhibitory effects on immune cells by various methods. Some strategies of tumor escape include causing dysfunction and apoptosis of T lymphocytes, causing dysfunction and apoptosis of dendritic cells (DCs), creating a loss of tumor recognition by immune cells, and creating resistance to immune intervention.[2] The basis of our research involving IL-12, GM-CSF, and Ig-4-1BBL recognizes some of the different mechanisms used by HNSCC to evade the immune system and attempts to counteract them.

The innate immune response is essential for the efficient eradication of tumor cells. Multiple factors coregulate this function including activated T lymphocytes, natural killer (NK) cells, and DCs. T cells require both T-cell receptor ligation and costimulation for optimal function. Without both factors, antigen-primed T cells will undergo apoptosis or become anergic. NK cells are regulated by the balance of

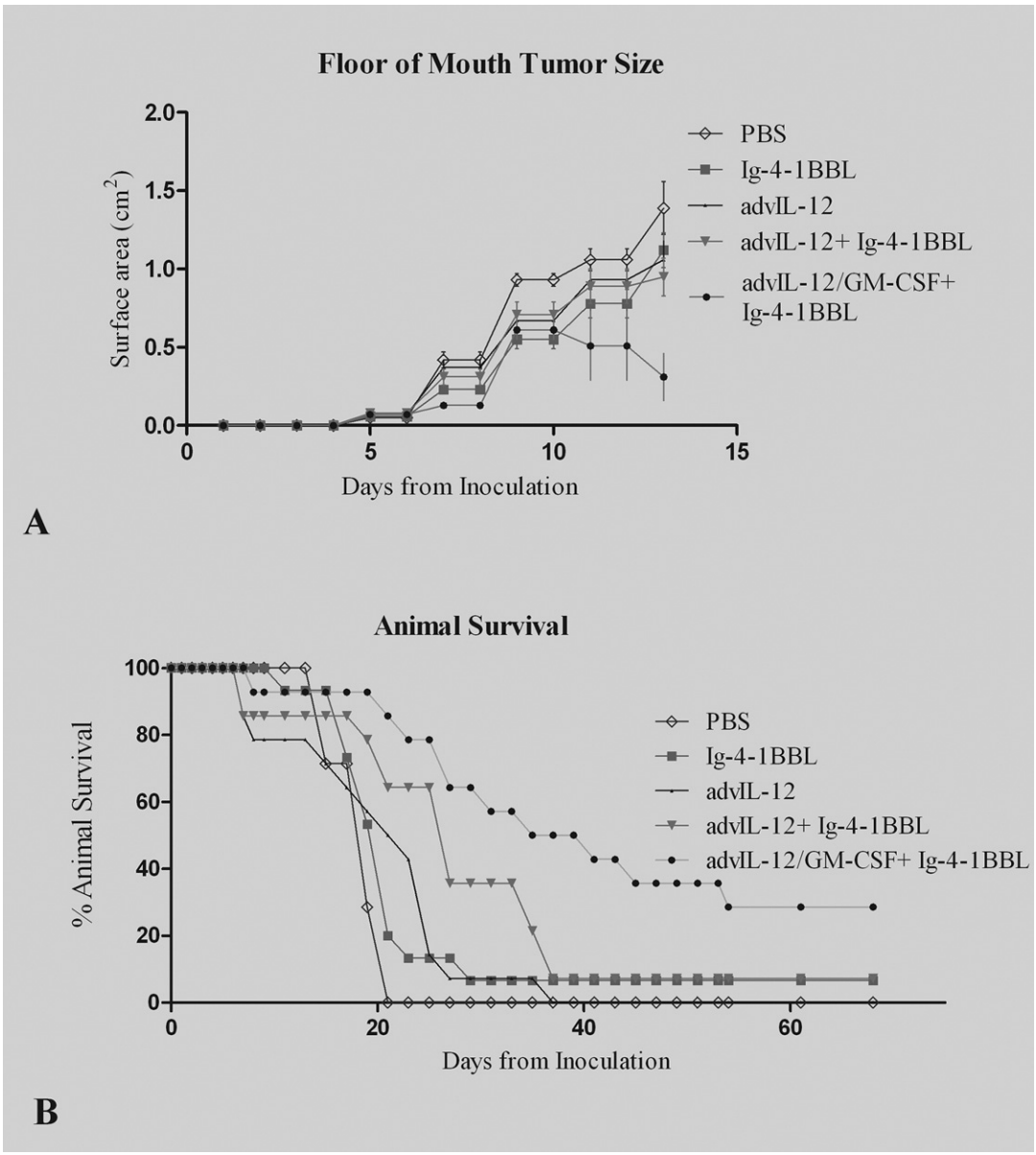

Figure 4 **(A)** The size of the FOM tumor (greatest length × width) after treatment with five daily injections (starting on postimplantation day 5) of respective vectors or PBS ± intraperitoneally inoculation of Ig-4-1BBL before initial control animal death. **(B)** Kaplan-Meier survival curves of mice with floor of mouth SCCA VII tumors after treatment with five daily injections (starting on postimplantation day 5) of respective vectors or PBS ± intraperitoneally inoculation of Ig-4-1BBL. After day 54, there were no further animal deaths until the conclusion of the study (day 230).

signals transmitted through cell surface-activating and -inhibitory receptors.[13,14] NK cell cytotoxicity is enhanced by multiple factors including IL-12.[15,16] In other forms of cancer, IL-12 has shown significant efficacy. Additionally, our prior research with vesicular stomatitis virus–expressing IL-12 showed increased survival in an FOM HNSCC animal model.[17]

GM-CSF is another cytokine recently studied for its promising antitumor effects. In vitro studies show an increased expression of DCs with GM-CSF–encoded viral vectors, and in vivo studies have provided significant antitumor responses with GM-CSF gene transfer in hepatic carcinomas and metastic colon cancers, although recent data by O'Malley et al[18] did not show enhanced effects in HNSCC when used in conjunction with IL-12 and herpes simplex virus thymidine kinase.[19] The ability of GM-CSF to augment cellular immunity is believed to result from the stimulation of the maturation and growth of antigen-presenting cells, thus improving antigen presentation.[2,3]

Other recent oncologic studies have shown promising results for Ig-4-1BB ligand[9-11]; 4-1BB is an inducible protein expressed on the surface of activated T lymphocytes, NK cells, and DCs. It is a member of the tumor necrosis factor family of receptors and is expressed on activated antigen-presenting cells. It can be activated either by its natural ligand or experimentally by an agonistic antibody,

which subsequently induces activation of the NF-kB, C-Jun NH2 terminal kinase/stress-activated protein kinase, and p38-signaling pathways. The 4-1BB–signaling pathway induces rescue of activated T cells from activation-induced cell death and leads to long-term survival of CD8T cells. In vivo, the activation of 4-1BB–signaling pathways has resulted in the regression of some tumors.[9-11]

The results of our study were extremely promising. The in vitro studies showed that tumor cells infected with advIL-12 produced a significant amount of IL-12 at 48 hours, whereas mock-infected tumor cells do not produce detectable levels of IL-12. Furthermore, tumor cells infected with advIL-12/GM-CSF produced a significant amount of GM-CSF at 24 hours and continued to produce more GM-CSF at 48 hours. Again, mock-infected tumor cells did not produce detectable levels of GM-CSF.

Additionally, our in vitro studies showed a greater than 10-fold increase in IL-12 expression with the advCMV-IL-12/GM-CSF vector versus advRSV-IL-12 vector ($P < 0.001$). The CMV promoter appears to be more effective in producing IL-12 than the RSV promoter. Other prior studies have shown an increase in local immune response with higher levels of IL-12.[8] Furthermore, because the goal of this study is to bring immunotherapy to clinical trials in head and neck cancer, we believed testing a viral vector carrying genes for both IL-12 and GM-CSF would be potentially beneficial in a future clinical setting, as a single agent, with its synergistic antitumoral effects. Clinically, a single agent would be easier to develop and administer than two separate agents.

Our in vivo studies provided additional support for future clinical application. Of the mice in the control group with FOM SCC, none survived past day 21. With the advIL-12 +Ig-4-1BBL, 7 percent of the mice with FOM SCC survived through to day 230 at the end of monitoring with no detectable cancer ($P < 0.006$ vs control). These data clearly showed a survival advantage in animals treated with double immunotherapy (advIL-12 +Ig-4-1BBL) over single therapy and control. More so, with the advIL-12/GM-CSF in combination with Ig-4-1BBL, 29 percent of the mice with FOM SCC survived through to the completion of the study (230 days, $P < 0.0001$ vs control) with no detectable cancer. When comparing the advIL-12/GM-CSF + Ig-4-1BBL with advIL-12 +Ig-4-1BBL, there was a strong trend toward survival advantage for advIL-12/GM-CSF + Ig-4-1BBL ($P < 0.06$ vs advIL-12 +Ig-4-1BBL). Although the P value for the triple immunotherapy was 0.06, the reduction in the size of the tumor was statistically significant ($P < 0.05$ when compared with advIL-12 +Ig4-1-BBL), and the median survival increased from 27 days with the double therapy to 38 days with the triple therapy. Further investigation into the triple therapy regimen is warranted to evaluate its efficacy in HNSCC patients.

In our animal models, no signs of toxicity were observed throughout the treatment period, up to 230 days. This includes both animals involved in the gene transfer and those injected with systemic Ig-4-1BBL. Animals showed no changes in daily weights or overall appearance (data not shown) when evaluating for signs of toxicity.

Additional investigations are necessary in two specific areas of immune therapy in order to enhance future investigations. First, future studies confirming the expression of cytokines in vivo after viral injection and evaluating lymphocytic (and other immune cell) infiltration of the tumor over time would further validate this immune-based treatment. The characterization of the functional role of these cells could further be confirmed by depletion studies (CD4, CD8, neutrophil, and/or macrophages). Second, studies evaluating the mechanism of the action of immune evasion by tumor cells in our model may provide insight into the survival advantage shown in this study. Currently, our study cured almost one-third of the treated mice. Based on this cure rate, it is likely that both additional cellular immune targets and differing mechanisms of immune modulation exist and their identification is critical to improve overall survival. With further characterization and increased understanding of immune response, we may be able to develop additional therapeutic agents to further increase the survival rate in future studies.

Chen et al[12] found that immunotherapy of advanced tumors often leads to only a transient tumor regression because of immunologic unresponsiveness to syngeneic tumors in hosts with large tumor burdens. They suggested that a state of tolerance, immune suppression, or both develop in larger, more advanced tumors.[20] Based on these results, an immune-based therapy may be particularly effective after conventional modalities, such as surgical, chemotherapy, and/or radiation therapy, which would initially reduce tumor volume.

At our institution, phase I trials have started using advIL-12 to treat breast and colon cancer metastases to the liver. In addition, we are currently developing clinical production of a human Ig-4-1BBL substrate with the goal of introducing phase I trials of Ig-4-1BBL in the near future to look at safety and efficacy alone and in combination with adv-IL-12. Our data provide support for the clinical development of an advIL-12/GM-CSF agent in combination with Ig-4-1BBL. Furthermore, in preclinical testing, we are in the process of developing a model to test the efficacy of our combined therapy in the setting of regional and distant metastases.

CONCLUSION

Our study shows a synergistic effect of IL-12, GM-CSF, and Ig-4-1BBL in combination with an adenoviral vector in producing a statistically significant reduction of tumor size as well as survival advantage in a murine FOM SCC model. The preclinical data support the use of advCMV-IL-12/GM-CSF + Ig-4-1BBL as a therapeutic agent in HNSCC.

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